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Deactivation of Akt by a small molecule inhibitor targeting pleckstrin homology domain and facilitating Akt ubiquitination

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The phosphatidylinositol-3,4,5-triphosphate (PIP3) binding function of pleckstrin homology (PH) domain is essential for the activation of oncogenic Akt/PKB kinase. Following the PIP3-mediated activation at the membrane, the activated Akt is subjected to other regulatory events, including ubiquitination-mediated deactivation. Here, by identifying and characterizing an allosteric inhibitor, SC66, we show that the facilitated ubiquitination effectively terminates Akt signaling. Mechanistically, SC66 manifests a dual inhibitory activity that directly interferes with the PH domain binding to PIP3 and facilitates Akt ubiquitination. A known PH domain-dependent allosteric inhibitor, which stabilizes Akt, prevents the SC66-induced Akt ubiquitination. A cancer-relevant Akt1 (e17k) mutant is unstable, making it intrinsically sensitive to functional inhibition by SC66 in cellular contexts in which the PI3K inhibition has little inhibitory effect. As a result of its dual inhibitory activity, SC66 manifests a more effective growth suppression of transformed cells that contain a high level of Akt signaling, compared with other inhibitors of PIP3/Akt pathway. Finally, we show the anticancer activity of SC66 by using a soft agar assay as well as a mouse xenograft tumor model. In conclusion, in this study, we not only identify a dual-function Akt inhibitor, but also demonstrate that Akt ubiquitination could be chemically exploited to effectively facilitate its deactivation, thus identifying an avenue for pharmacological intervention in Akt signaling.

chemical screening | cell death

A number of pathogenic conditions, including cancer, arise from perturbation of intracellular signaling pathways that control the amount of phosphatidylinositol-3,4,5-triphosphate (PIP3) at the membrane. PIP3 level is mainly controlled positively by PI3K and negatively by lipid phosphatase PTEN (1, 2). PIP3 exerts its functions by mediating the recruitment of various signaling effectors at the membrane. The serine/threonine protein kinase Akt (also known as PKB) is one of the major downstream effectors recruited by PIP3. Akt contains an N-terminal pleckstrin homology (PH) domain, which specifically binds to PIP3, enabling its membrane translocation and subsequent activation by upstream kinases (3,4). The activated Akt, in turn, phosphorylates a variety of proteins involved in diverse cellular processes, including cell proliferation and survival (5).

Because of its crucial involvement in tumorigenesis and drug resistance of cancer cells, the PIP3/Akt pathway has been the primary target for anticancer drug development (5, 6). Various current efforts to inhibit Akt function are mainly focused in two aspects: reducing PIP3 level and inhibiting Akt activity (7–11). We also showed that two inositol phosphates, InsP7 and Ins (1,3,4,5)P4, compete with PIP3 for binding to Akt PH domain (12, 13). Additionally, recent studies revealed that the ubiquitination of Akt plays important regulatory functions (14–16), providing a potential new avenue for pharmacological intervention. In this study, we identified and characterized a dual-function Akt inhibitor that directly facilitates Akt ubiquitination and deactivation. We demonstrated its efficacy toward a cancer-relevant

and PI3K inhibitor-resistant Akt1 (e17k) mutant. As a result of its dual inhibitory activity, SC66 manifests a more effective growth suppression of transformed cells compared with other inhibitors of PIP3/Akt pathway. Therefore, this study offers validation for the chemical-assisted ubiquitination as a legitimate strategy to terminate Akt signaling.

Results

Cell-Based Screening Identifies a Compound that Directly Facilitates Akt Ubiquitination.

To better understand the regulatory mechanisms of the PIP3/Akt pathway, we carried out an image-based chemical screening by using the spatial distribution of Akt1 PH domain/EGFP fusion protein (PH-EGFP) as a read-out (to be described elsewhere). This screening identified a group of 12 chemicals (termed group II) that not only prevented the membrane translocation of PH-EGFP, but also induced its accumulation into a subcellular location reminiscent to the pericentrosomal region (Fig. S1 and Dataset S1). Interestingly, the compounds SC13, SC66, and SC67 contain a pyridine moiety that is also found in some chemicals known to inhibit Akt (17, 18). In this study, we focused on characterizing SC66 as a representative of this group of compounds. First, we confirmed that this subcellular location indeed represented the pericentrosomal region by immunostaining with γ -tubulin, a centrosomal marker (Fig. 1A). The SC66-induced pericentrosomal accumulation was specifically mediated by Akt PH domain, as EGFP alone or EGFP fused to PH domain from PLC- δ had no effect (Fig. 1A). Other group II compounds also showed no effect on the membrane localization of PH-PLC δ -EGFP (Fig. S2). The level of PIP3 at the membrane did not affect the SC66-induced pericentrosomal localization, as cotreatment with IGF1 or PI3K inhibitor failed to yield any differential effects. Likewise, a PIP3-nonbinding mutant PH (r25c)-EGFP was also accumulated in the pericentrosomal region. As revealed by colocalization with PH-EGFP, the full-length Akt1 could be also accumulated in this region by SC66 and other group II compounds (Fig. 1A and Fig. S3). To test if SC66 could inhibit the Akt signaling pathway, HEK293T cells, which were shown to contain a high level of PIP3 (19), were treated with different amounts of SC66, and the whole-cell lysates were examined for the phosphorylation level of Akt and its known target proteins (Fig. 1B). At a concentration that led to the pericentrosomal accumulation, SC66 significantly reduced the phosphorylation level of both Akt and its targets, but not those of other cellular kinases. Importantly, unlike the Akt phosphorylation at S473, the phosphoryla-

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The authors declare no conflict of interest.

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were injected with SC66 twice per week and the size of tumors was measured every 3 d for 21 d. Compared with vehicle alone, SC66 led to a significant inhibition of tumor growth, confirming the anticancer property in vivo (Fig. 4C).

Discussion

In this study, we identified a group of chemicals that inhibit Akt activation through interfering with PH domain binding to PIP₃, and lead to pericentrosomal localization of Akt. Altering the spatial distribution of Akt can lead to functional perturbation by affecting substrate recognition and facilitating dephosphorylation. Elucidating the mode of action of these compounds will undoubtedly provide important new insights into the regulatory mechanisms of oncogenic PIP₃/Akt signaling pathway and the development of new therapeutic strategies. We extensively characterized a pyridine-based allosteric Akt inhibitor, SC66, that directly facilitates Akt ubiquitination in vitro and in vivo. We elucidated the mechanisms of its dual inhibitory function, identified the efficacy toward a cancer-relevant and PI3K inhibitor-resistant Akt1 (e17k) mutant, and demonstrated the synergistic apoptotic activity with the PI3K inhibitor and the in vivo anticancer efficacy as a single agent. We also showed that, because of its unique dual inhibitory activity, SC66 manifested a more effective growth suppression of transformed cells compared with other inhibitors of PIP₃/Akt pathway.

The phosphorylated Akt was found to be ubiquitinated in an in vitro assay. Intriguingly, the phosphorylated and ubiquitinated Akt could be hardly detectable in lysates from cells treated with SC66.

Inhibition of initial phosphorylation by preventing Akt membrane translocation may explain this finding. However, given its efficacy toward Akt dephosphorylation in HEK293T cells, which contain a high level of PIP₃, also indicates other possibilities. For example, the phosphorylated Akt, when bound to SC66, might be rapidly dephosphorylated and/or the ubiquitinated Akt by SC66 might be less likely to be phosphorylated. This prediction would be consistent with its inhibitory effects toward Akt1 (e17k) mutant, which is “membrane-prone” independent of PIP₃. Further studies, including the identification of cellular factors involved in SC66-mediated Akt ubiquitination, are needed to clarify these issues. As such, SC66 represents a unique chemical tool to investigate the mechanisms of ubiquitination-dependent Akt regulation in physiological and stressed conditions.

Materials and Methods

Cell Culture and Stable Cell Lines. For routine maintenance, all cell lines were cultured in medium supplemented with 10% FBS and 1% penicillin and streptomycin under 5% CO₂. HEK293, HeLa, and their derivative cell lines were maintained in DMEM. NB4 and HS-Sultan cells were cultured in RPMI medium. HeLa cell lines stably expressing PH-EGFP were described previously (30). Other stable HEK293 cell lines expressing Akt1 mutants, Akt 3, or PH-EGFP were generated by transfecting the corresponding expression plasmids and selected and maintained in the presence of G418 (Invitrogen).

Time-Lapse Live Cell Imaging for Spatial Distribution of EGFP Fusion Proteins.

HeLa cells transfected with the plasmids encoding the EGFP fusion proteins were plated into a 35-mm glass-bottom dish (MatTek) and cultured for 24 to 48 h before imaging. For PH-EGFP membrane translocation assay, cells were serum-starved in 2 mL Leibovitz L15 medium for 1 to 2 h, which was replaced with 1 mL of fresh serum-free Leibovitz L15 medium containing each compound. After 30 min incubation, IGF1 (5 ng/mL) was added and an image was taken every 5 to 10 min under a 40× oil objective lens. The relative fluorescent intensity at the membrane versus adjacent cytoplasm (for PH-EGFP) or cytoplasm versus nucleus (for EGFP-Foxo) was determined. Western blot and immunostaining, PIP₃ ELISA, in vitro PIP₃ binding, in vitro ubiquitination assay, time-lapse live cell imaging analysis for mitotic and apoptotic cells, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and other related assays are described in *SI Materials and Methods*.

Mouse Xenograft Experiment. Eight-week-old female NOD/SCID mice were used in this study. Fifteen mice received an s.c. injection of 2×10^6 293T cells in the both flanks. Seven days after injection, mice were randomized into three groups ($n = 5$ mice per group) to receive vehicle (control) or SC66 15 mg/kg or 30 mg/kg i.p. SC66 dissolved in DMSO was further diluted in 0.2 mL of PBS solution containing 25% ethanol for i.p. injections. SC66 was administered twice per week (total of six times). The size of tumor was measured three times per week by using a caliper, and mice were killed on day 28 after the injection of cancer cells. The tumor volumes were calculated as length \times width² \times 0.52. Data are presented as the mean value. A Student *t* test was performed to evaluate the difference between mean values. $P < 0.05$ was considered to indicate a statistically significant difference.

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Supplementary Table I. Characterization of Group II compounds.

ID	Mol. Formula	M. W.	Conc. ^a μM at 8 μg/ml	Membrane translocation / localization assay ^b						PIP3 binding ^c Inhibition of PH domain binding to PIP3 beads	Western blot ^d Effect on pAkt level drug conc. (8 μg/ml)			EGFP-Foxo ^e Nuclear Foxo after IGF
				Akt PH		Live cell imaging			PLCβ-PH Live cell 8 μg		HeLa	NB4	HsSultan	
				High throughput	Second	16 μg	8 μg	4 μg						
SC1	C ₉ N ₄ O ₄	191	42	+	+	+	+	+	-	+/-	+	+	+	++
SC11	C ₁₈ H ₁₄ N ₂ O ₅	338	24	+	+	+	+	+	-	+	+	+	+	++
SC13	C ₂₁ H ₁₇ N ₂ O ₃ SBr	456	18	+	+	+	+	+	-	++	+	n.d.	n.d.	++++
SC19	C ₁₄ N ₂ OCl ₂	282	28	+	+	+	+	+	+/-	+++	+	+	+	++
SC23	C ₁₈ H ₁₄ N ₄ O ₃ S	326	25	+	+	+	+	+	-	+++	+	+	-	++++
SC27	C ₁₀ H ₇ NOS	189	42	+	+	+	+	+	-	++	+	+	-	++++
SC49	C ₁₈ H ₁₁ N ₃ O ₆	365	22	+	+	+	+	+	-	+	+	+	+	+++
SC63	C ₁₃ H ₁₂ N ₂ O ₂ S ₂	292	27	+	+	+	+	+	-	++	+	+	+	+
SC66	C ₁₈ H ₁₈ N ₂ O	276	29	+	+	+	+	+	-	+++	+	+	+	++
SC67	C ₁₈ H ₁₇ N ₃ O	291	27	+	+	+	+	+/-	-	++	+	+	+	++
SC86	C ₁₈ H ₁₆ N ₄ O ₂ S ₂	384	21	+	+	+	+	+	-	+	+	n.d.	n.d.	+
E26	C ₁₁ H ₈ NO ₃	203	39	+	+	+	+	+	-	++	+	+	+	+

Supporting Information

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SI Discussion

Deactivation of PIP3/Akt signaling pathway suppresses the growth of tumors and the resistance of cancer cells to chemotherapeutic drugs (1–4). The signaling pathways for PIP3 production and the mechanisms of PIP3-mediated Akt activation are well established and broadly applicable to different cell types. However, how the activated Akt is deactivated in specific cellular contexts remains to be elucidated. Facilitating the deactivation of Akt would enhance the efficacy of various inhibitors of PI3K, which prevent only the activation step. Inhibiting the kinase activity of Akt will be an obvious choice. However, as elegantly demonstrated in a recent study (5), paradoxically, chemicals targeting the Akt kinase domain were shown to lead to the “inhibitor-induced Akt activation,” raising concerns for the long-term clinical utility of such inhibitors (5).

Akt is known to undergo dynamic conformational changes. Controlling the structural integrity of Akt appears to serve as another regulatory mechanism. For example, it has been reported that mTorC2, apart from its activity as an S473 kinase, plays important roles in maintaining the structural integrity and maturation of Akt by phosphorylating at T450 in turn motif (6, 7). Lack of phosphorylation at this site, as a result of genetic ablation of mTorC2 components, results in the structural instability of Akt, leading to an increased susceptibility to proteasome-dependent degradation. We showed that, compared with WT, Akt1 (e17k) was unstable upon inhibition of HSP90. However, this instability did not appear to be caused by the lack of phosphorylation at T450. A recent study reported an enhanced ubiquitination of Akt1 (e17k) mutant by TRAF6 E3 ubiquitin ligase, which facilitates its membrane localization and activation (8). We also found a faster kinetics of SC66-induced ubiquitination of this mutant.

How this mutation in PH domain leads to an enhanced Akt ubiquitination is not clear. Further studies, including the identification of cellular factors involved in SC66-induced Akt ubiquitination, are needed. Ectopic expression of TRAF6 or CHIP E3 ubiquitin ligases known to be involved in Akt ubiquitination (8, 9), failed to affect the SC66-induced Akt ubiquitination. Also, as SC66 inhibits Akt phosphorylation, the drug-bound Akt is unlikely to be directly ubiquitinated by TTC E3 ubiquitin ligase, which was shown to specifically bind to and ubiquitinate the phosphorylated Akt (10).

A wide variety of PI3K inhibitors have been developed and are continuously being identified. One caveat of suppressing PIP3 signaling in cancer cells by PI3K inhibitor alone is the activation of compensatory mechanisms, as demonstrated in HeLa cells treated with LY294002 or wortmannin. Likewise, targeting the Akt activity alone can be compensated by other AGC family member kinases. Considering the heterogeneity and various genetic lesions of cancers, the effective termination of Akt signaling requires a multifaceted strategy that prevents the membrane translocation and facilitates its deactivation. The dual-function allosteric inhibitor elucidated in this study exemplifies one such new strategy.

SI Materials and Methods

Reagents and Antibodies. Plasmids encoding human Akt1 were initially obtained from Dana–Farber/Harvard Cancer Center DNA Resource Core and subcloned into the pcDNA3.1/V5-His-TOPO vector. The site-directed mutagenesis was done with the QuikChange mutagenesis kit (Stratagene). EGFP-Foxo1 was obtained from Addgene. All Akt- and phosphorylation-specific antibodies were purchased from Cell Signaling Technology; V5

antibody was from Invitrogen; ubiquitin antibody (sc-9133) was from Santa Cruz Biotechnology. All other reagents, including the HRP-conjugated secondary antibodies, for Western blot were from GE Healthcare. LY294002, Akt inhibitors, rapamycin, and wortmannin were purchased from EMD Biosciences; PI-103, PIP3-coated beads, and PIP3 ELISA kit were from Echelon.

Western Blot and Immunostaining. Preparation of cell lysates, SDS/PAGE, and Western blot, and other standard molecular biological techniques, were essentially the same as described previously (11). For immunostaining of Akt1 (1:2,000 for V5 antibody) and phospho-Akt (1:200 for pS473), cells were fixed in 3% paraformaldehyde, and followed the same procedure as previously described (12).

PIP3 ELISA and in Vitro PIP3 Binding Assay with Purified PH-EGFP Protein. The serum-starved HeLa cells (1×10^7) were pretreated with LY294002 (20 μ M) or group II chemicals (4 μ g/mL) for 30 min, then stimulated with IGF1 (5 ng/mL) for 20 min. Extraction of PIP3 by sequential centrifugation in methanol:chloroform:HCl buffer and measurement of the extracted PIP3 was done using the PIP3 Mass ELISA Kit (K-2500s; Echelon), according to the instructions. For purification of PH-EGFP protein, HEK cells (1×10^8) stably expressing PH-EGFP tagged with the C-terminal V5/His were suspended in PBS solution containing 0.3% CHAPS, 20 mM imidazole, and protease inhibitor mixtures. The cell suspension was frozen on dry ice for 30 min and thawed at room temperature. The lysates were cleared by centrifugation and loaded on the column packed with Ni-NTA beads (Qiagen). After washing three times in PBS solution containing 0.3% CHAPS and 50 mM imidazole, the bound fraction was eluted with 100 mM imidazole. The eluted protein was concentrated in the binding buffer (10 mM Hepes, pH 7.4, 0.25% Nonidet P-40, 150 mM NaCl, 0.5 mM β -mercaptoethanol) by centrifugation (Amicon Ultra 10K cutoff filter; Millipore). The purified PH-EGFP protein (800 ng/mL) was preincubated with group II compounds (1 μ g/mL) for 20 min on ice, and incubated with 20 μ L of PIP3-coated beads (Echelon) for overnight at 4 $^{\circ}$ C. After washing the beads three times with binding buffer at room temperature, the amount of bead-bound PH-EGFP protein was determined by Western blot.

Proteasome, Deconjugation, and in Vitro Ubiquitination Assay Using Cell Lysates. HEK293 cells (1×10^6) were treated with compounds (4 μ g/mL) for 1 h. After washing with PBS solution, the cell pellet was lysed on ice for 15 min in 200 μ L lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM β -glycerophosphate, 1% Triton X-100). After clearing cell debris by centrifugation at 4 $^{\circ}$ C, the extract (50 μ L) was subjected to proteasomal activity using Proteasome-Glo Chymotrypsin-Like Assay (G8621; Promega). The same extract was also assayed for deconjugation activity by using DUB-Glo Protease Assay (G6260; Promega). For in vitro ubiquitination assay, HEK293 expressing Akt1 tagged with V5/HIS were lysed in a buffer containing 50 mM Hepes, pH 7.4, 0.2% Nonidet P-40, 0.5 mM β -mercaptoethanol, and protease inhibitor mixtures. The lysates were frozen on dry ice for 30 min and thawed at room temperature. After centrifugation, the extract was subjected to in vitro ubiquitination reaction. Typically, 100 to 200 μ g of total proteins were mixed with 1 μ g of chemicals on ice for 10 min, and supplemented with MG132 (5 μ M), ubiquitin aldehyde (4 μ M), and ATP (5 mM) in 50 μ L of ubiquitin conjugation reaction

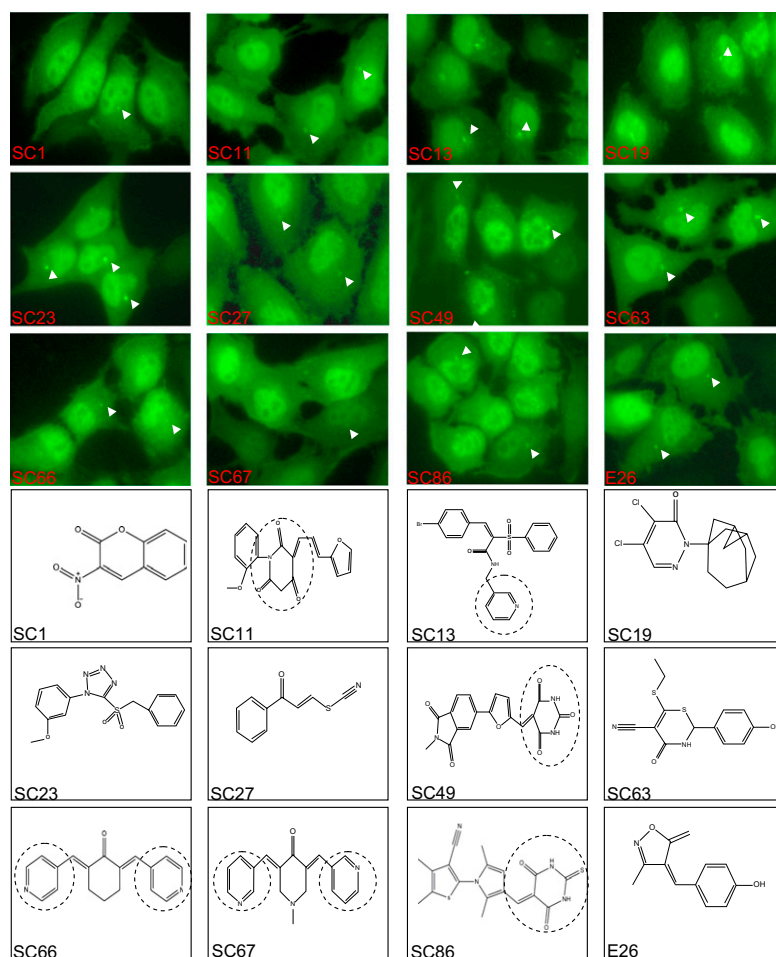


Fig. S1. Representative pictures of live imaging of HeLa-PH-EGFP cells treated with group II compounds (chemical structures, *Bottom*). Arrows indicate the pericentrosomal region. Dotted circles indicate the barbiturate-derivative and pyridine moiety.

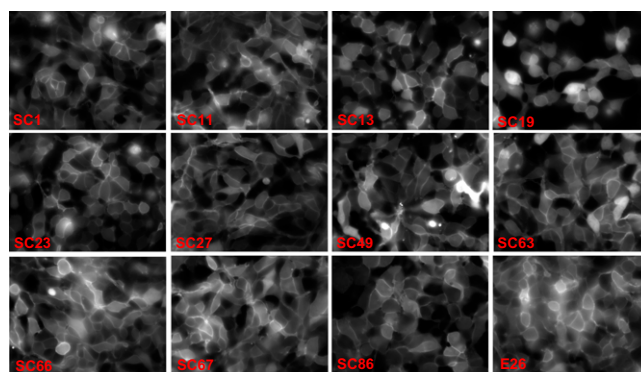
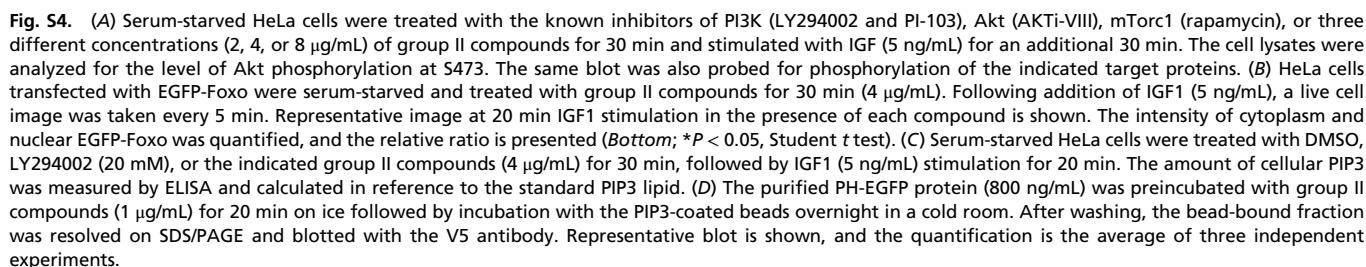
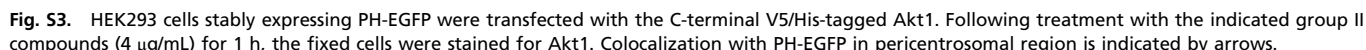


Fig. S2. The effect of group II compounds (8 $\mu\text{g/mL}$) on the PtdIns(4,5)P₂-mediated membrane localization of EGFP-PLC- δ 1-PH domain.



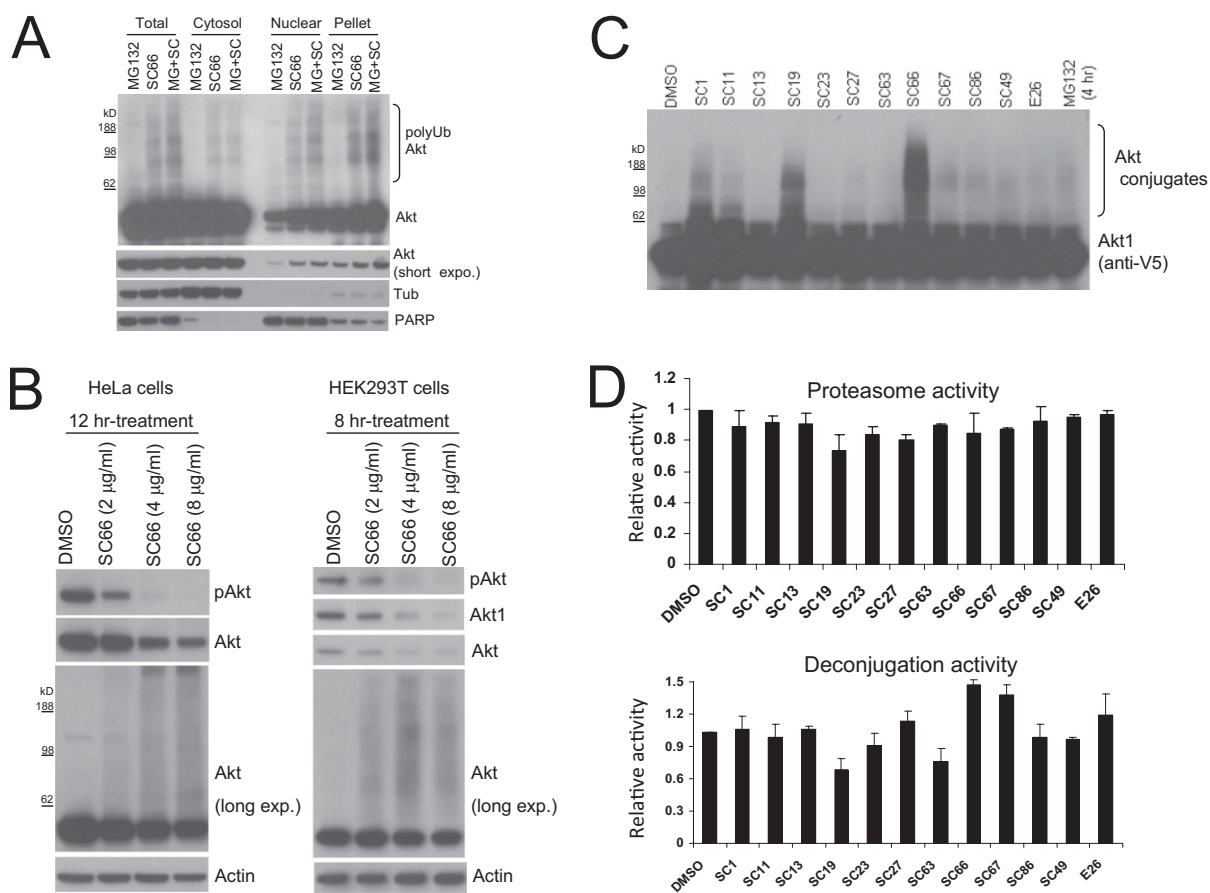


Fig. S5. (A) HeLa cells were pretreated with MG132 (10 μ g/mL) for 2 h before addition of SC66 (4 μ g/mL). Following an additional 2-h incubation, the total cell extract, cytosolic, nuclear, or pellet (insoluble) fraction were analyzed for Akt. The same fractions were simultaneously blotted for other cellular proteins: β -tubulin for cytosolic and PARP for nuclear fractions, respectively. (B) HeLa or HEK293T cells were treated with different amounts of SC66 for the indicated time points and the levels of pAkt, Akt, and actin were analyzed. (C) HEK293 cells stably expressing Akt1, HEK293-Akt1, were treated with group II compounds (4 μ g/mL) for 1 h or MG132 (10 μ g/mL) for 4 h, and the cell lysates were analyzed for Akt1 by Western blot with a monoclonal V5 antibody. (D) Proteasomal and deconjugation activity from the cytosolic cell lysates were measured. Relative activity in reference to DMSO-treated cells is presented.

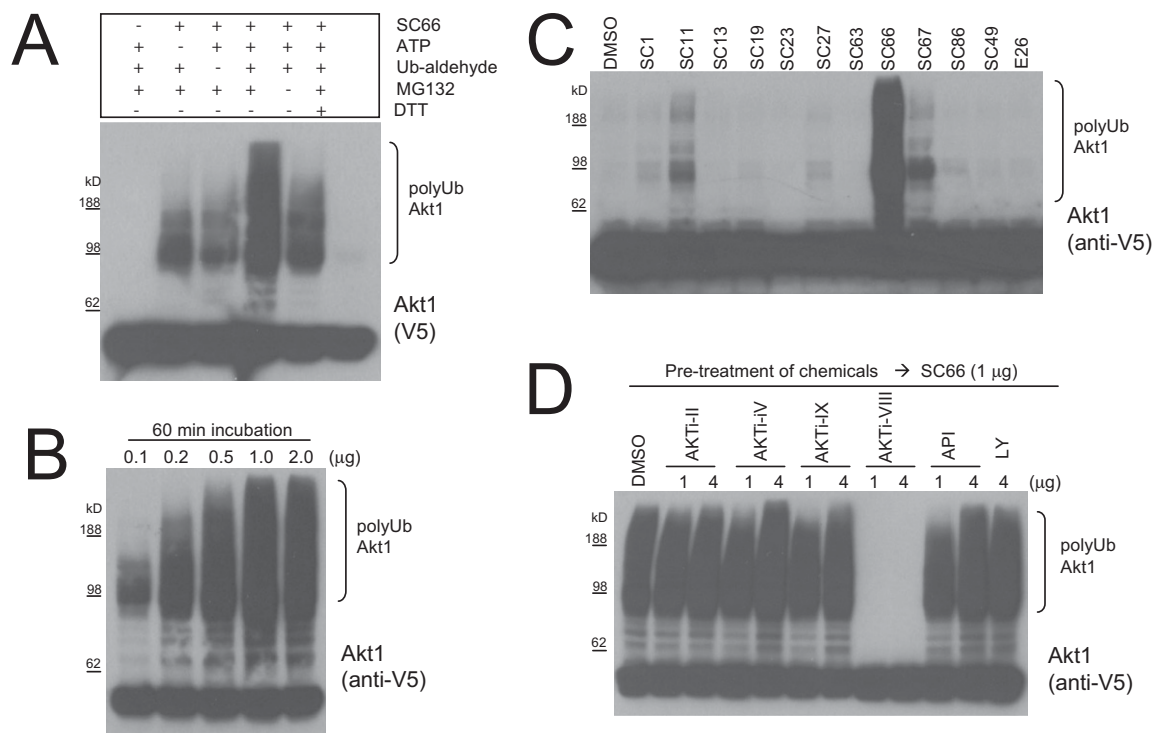


Fig. S6. (A) SC66-induced in vitro ubiquitination assay for Akt. HEK293-Akt1 cell lysates were incubated with the indicated combinations of ATP, ubiquitin aldehyde, MG132, DTT, and SC66 for 1 h. The absence of ubiquitin aldehyde, which inhibits deubiquitination, most significantly affected the Akt ubiquitination. The ubiquitinated Akt detected in the absence of additional ATP could be a result of residual ATP and preformed E1- and E2-ubiquitin complex present in the cell extract. In the presence of DTT, which disrupts the thioester bond between E1- and E2-ubiquitin that is required for the subsequent ubiquitination by E3 ligases, the SC66-induced Akt ubiquitination was almost completely abolished. (B) SC66 dose-dependent in vitro ubiquitination of Akt. (C) The effect of group II compounds on the in vitro ubiquitination of Akt1. (D) The effects of AKTi-VIII and other chemicals known to inhibit Akt pathway on in vitro ubiquitination of Akt1 by SC66. The indicated amounts of chemicals were pre- or simultaneously incubated with SC66, followed by in vitro ubiquitination reaction.

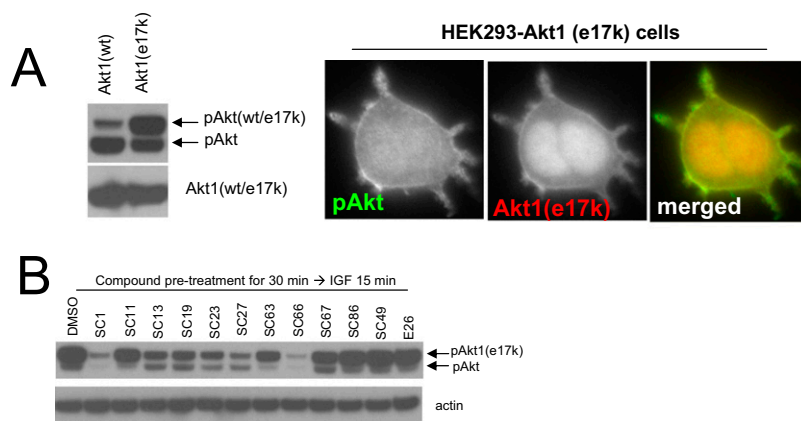


Fig. S7. (A) Level of phospho-Akt and cellular localization of Akt1 (e17k). (B) Inhibitory effects of group II compounds (8 μg/mL) on the phosphorylation of Akt1 (e17k).

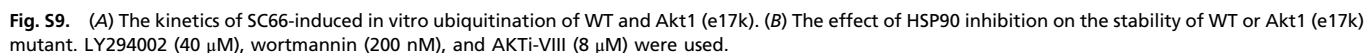
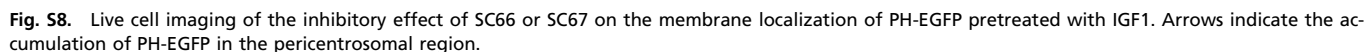




Fig. S11. (A) Quantification of cell migration in Fig. 3A. The relative migration area compared with DMSO control was presented. $*P < 0.05$ by Student t-test. (B) A schematic presentation of the quantification of HeLa cell migration in Fig. 3A.





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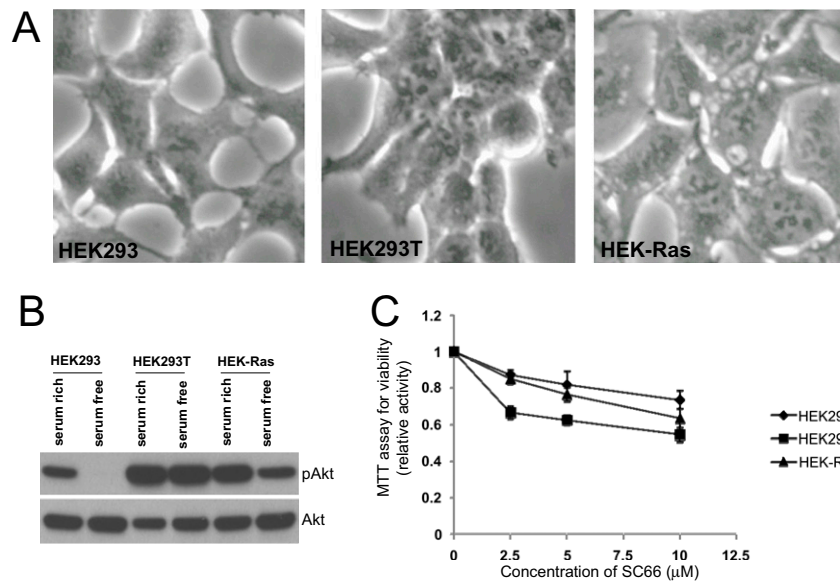
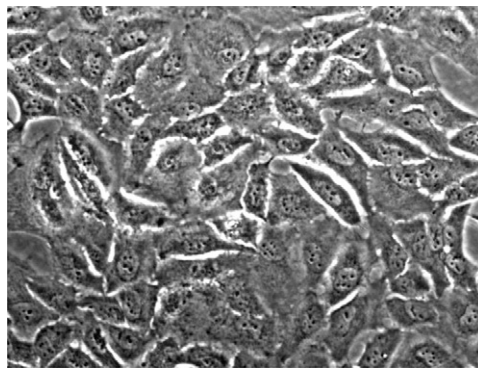
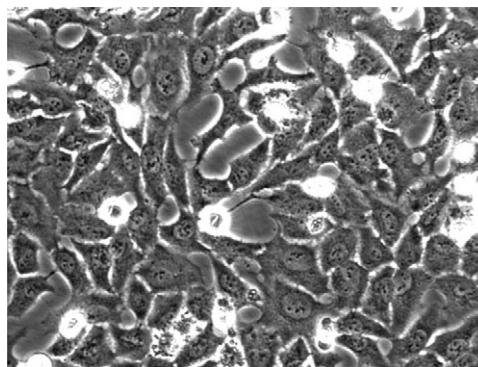


Fig. S14. (A) Morphology of the parental HEK293 cells and cells transformed by SV40 large T antigen (HEK293T) or H-Ras oncogene (HEK-Ras). (B) The level of phosphorylated Akt in cells grown in serum-rich or serum-free (for 1 h starvation) medium. (C) Differential growth-suppressive effects of SC66 on parental HEK cells and transformed cells. The relative viability was determined after 24 h treatment with SC66.



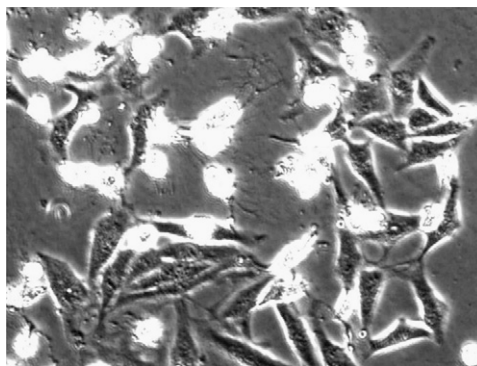
Movie S1. Live cell imaging of HeLa cells treated with SC66 (2 μg/mL). Frames were taken every 15 min for 14 h.

[Movie S1](#)



Movie S2. Live cell imaging of HeLa cells treated with LY294002 (20 μM). Frames were taken every 15 min for 14 h.

[Movie S2](#)



Movie S3. Live cell imaging of HeLa cells treated with SC66 and LY294002. Frame were taken every 15 min for 14 h.

[Movie S3](#)

Dataset S1. Characterization of group II compounds

[Dataset S1 \(XLS\)](#)

^a8 $\mu\text{g/mL}$ is the equivalent to 1 \times concentration of the initial high-throughput screening.

^bPlus signs indicate inhibition of membrane translocation greater than 50% of control.

^cNumber of plus signs represents the relative inhibitory activity compared with DMSO control.

^dPlus signs indicate inhibition of Akt phosphorylation (s473) greater than 50% of control.

^eNumber of plus signs represents the relative intensity of nuclear EGFP-Foxo over cytoplasm.